Interesting Measure = DNA adducts

Effects of a 6-Month Vitamin Intervention on DNA Damage in Heavy Smokers[1]

poly aromatic hydrocarbons

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Abstract

Because their formation is associated with tumor development in specific tissues, DNA adducts have potential usefulness as intermediate end points in chemoprevention studies. To determine the efficacy of a combination of antioxidant vitamins (vitamins C and E and β-carotene), a randomized clinical trial was conducted among heavy smokers using DNA damage as the end point. Immunological methods were used to measure polycyclic aromatic hydrocarbon-DNA adducts and oxidative DNA damage (8-oxo or hydroxydeoxyguanosine) in mononuclear and oral cells. A total of 121 subjects were randomized to the 6-month intervention and received either vitamins or placebo. Dropout rates were higher in the placebo than in the vitamin group; 65% of subjects in the vitamin group, but only 47% in the placebo group, provided specimens at 6 months. Plasma levels of all three antioxidants rose significantly in the vitamin group but not in the placebo group. All four measures of DNA damage decreased in both groups; the between-group differences were not statistically significant. These data do not provide clear evidence that antioxidant vitamin intake prevents DNA damage. However, the study demonstrates that DNA damage is a useful end point in chemoprevention trials.

Introduction

The evidence that cigarette smoking causes cancer is now overwhelming. The burning of tobacco is known to generate carcinogenic PAHs,[3] aromatic amines, and tobacco-specific nitrosamines (1). Virtually all PAH mixtures contain BP, which can therefore serve as a marker of total PAH exposure. Metabolic activation of BP to a diol epoxide results in binding to DNA, which has genotoxic and carcinogenic effects (reviewed in Refs. 2 and 3). In general, DNA adduct formation is correlated with the development of tumors in a specific tissue; adduct formation is considered necessary but not sufficient for tumor initiation (4).

Cigarette smoke also contains many compounds that can generate oxidative DNA damage (1). In addition, metabolism of many chemical carcinogens such as BP also results in the generation of oxidative stress and oxidative DNA damage (5). 8-Oxo, or 8OHdG, is recognized as a useful marker for oxidative DNA damage because it is one of the most abundant and is also mutagenic (6).

Evidence from a variety of sources suggests that components of the human diet can impede carcinogenesis by scavenging oxygen radicals or interfering with the binding of carcinogens to DNA (7). In the past decade, several intervention trials have been undertaken to test the hypothesis that antioxidant vitamin supplements could reduce cancer risk. β-Carotene was, until recently, the micronutrient of greatest interest. However, two randomized clinical trials of β-carotene to reduce lung cancer risk in heavy smokers were terminated early in the mid-1990s because interim analyses indicated that lung cancer incidence was higher in the treatment group than in the placebo group (8, 9). A third intervention reported no effect of β-carotene on lung cancer in smokers (10).

Since then, investigators have tried to account for these unexpected findings and have continued to explore the potential effects of β-carotene and other micronutrients on cancer risk. One hypothesis about the failure of the α-Tocopherol and β-Carotene Cancer Prevention and β-Carotene and Retinol Efficacy trials is that the carcinogenic process in the study participants was too far advanced for β-carotene to be beneficial (11). Another is that the β-carotene dose was too far above physiological levels and therefore had or induced pro-oxidant activity (11).

We hypothesized that antioxidants might be most beneficial in the early stages of carcinogenesis, such as the initiation phase of DNA adduct formation. In cross-sectional studies, we had found an inverse relationship between lymphocyte PAH-DNA adduct levels and serum levels of vitamins C and E (12). A relationship between adduct levels in mononuclear cells and lung tissue has also been observed (13, 14). Other studies had found that lung cancer is associated with high adduct levels (15–18) and low serum vitamin levels (19–21). We therefore initiated a randomized, placebo-controlled trial to test the efficacy of an antioxidant vitamin supplement in reducing DNA damage (PAH-DNA and 8OHdG) among heavy smokers.

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[3] The abbreviations used are: PAH, polycyclic aromatic hydrocarbon; BP, benzo(*a*)pyrene; CI, confidence interval; 8OHdG, 8hydroxy or oxodeoxyguanosine; CV, coefficient of variation; HPLC, high-performance liquid chromatography; GSTM1, glutathione *S*-transferase M1.

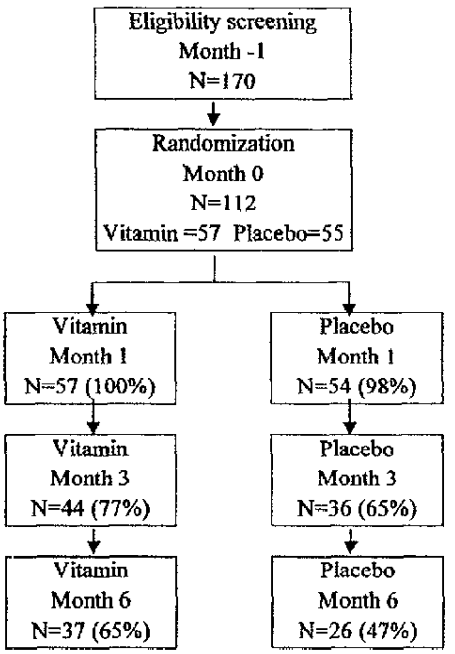

Fig. 1. Study participation.

Materials and Methods

Recruitment. We recruited study participants by posting signs around Columbia Presbyterian Medical Center inviting eligible individuals (adults 18 years of age and older who smoked one or more packs of cigarettes per day and were not currently taking the study vitamins) to telephone for an appointment. Exclusion criteria were nondetectable PAH-DNA adduct levels in mononuclear cells and plasma vitamin levels higher than 1.0 mg/dl for vitamin C, 15 μg/dl for β-carotene, and 1.2 mg/dl for α-tocopherol at the first study visit.

Study Procedures. The study was a randomized, placebo-controlled, double-blind trial of an antioxidant vitamin supplement. At the first study visit (screening, -1-month time point), we obtained informed consent; administered a baseline questionnaire about demographic factors, diet, personal health, and smoking habits; collected blood (45 ml), urine (~100 ml), and oral cell specimens; and provided each study participant with a 1-month supply of the placebo (Fig. 1). Before the second visit (baseline, 0-month time point), each individual was assigned randomly to treatment or placebo. The method of randomly permuted blocks (22) was used for treatment assignment.

At the second visit, study participants returned the run-in bottle and remaining placebo tablets and received a 1-month supply of treatment or placebo; they also provided baseline blood, urine, and oral cell specimens for the trial. Once a month for the next 6 months, study participants returned to exchange the bottle received at the previous visit for a new bottle. Blood, urine, and oral cell specimens were collected at the 1-month, 3-month, and 6-month follow-up visits. Subjects received monetary compensation when providing biospecimens. Transportation costs were also provided to enhance subject retention.

Intervention. Each tablet bottle contained 75 active treatment or placebo tablets. We asked the study participants to take one tablet with the first meal of the day and another, with food, before bedtime. Each active treatment tablet contained 250 mg of vitamin C, 200 IU of α-tocopherol, and 6 mg of β-carotene. Study participants were telephoned during the week before each follow-up visit to confirm the date and time of the next appointment and to identify any problems or side effects associated with study participation.

Biospecimen Collection. Blood was collected into Vacutainers containing EDTA and kept under subdued light until isolation of mononuclear cells by centrifugation over Histopaque 1077 (Sigma Chemical Co., St. Louis, MO). Some plasma aliquots were stored with an equal volume of 10% metaphosphoric acid for vitamin C analysis.

Oral cells were collected by rinsing the mouth with PBS after a brief rinsing with water to remove food particles. Microscope slides were precoated by dipping in 3-aminopropyltriethoxysilane (6 ml in 300 ml of acetone), rinsing in acetone twice (2 min each), and then in water twice (2 min each). Cells were collected by centrifugation at 1000 rpm for 10 min and the pellet resuspended in sucrose buffer [0.25 M sucrose, 1.8 mM $CaCl_2$, 25 mM KCl, 50 mM Trisma Base (pH7.5)]. The oral cell suspension (30–50 μl) was added to 300 μl carbowax-ethanol buffer (1 ml of 60% polyethylene glycol 1000 and 40% water added to 74 ml 70% ethanol), vortexed briefly, and 150 μl added to each of two cytofunnels. The samples were spun at 300 rpm for 5 min on a Cytospin 3 (Shandon, Pittsburg, PA), air-dried for 10–30 min, fixed in 95% ethanol (-20°C for 10 min), and stored at -20°C until stained. A similar procedure was used to prepare slides of isolated mononuclear cells after they were washed once with PBS.

Laboratory Analysis. Baseline through 6-month samples from the same individual were batched for analysis with the laboratory blinded to treatment status and time point.

PAH-DNA in Mononuclear Cells by ELISA. DNA was isolated from mononuclear cells by RNase and proteinase K treatment, extraction with chloroform/isoamyl alcohol, and ethanol precipitation. PAH-DNA adducts were analyzed by competitive ELISA essentially as described previously, using polyclonal antiserum no. 29 (23). For analytical purposes, those samples with <15% inhibition were considered nondetectable and assigned a value of $1/10^8$, an amount midway between the lowest positive value and zero. Antiserum no. 29 (24), generated from a rabbit immunized with benzo(*a*)pyrene diol epoxide-DNA, cross-reacts with the diol epoxide DNA adducts of several other PAHs (25). Thus, this assay detects multiple PAH-DNA adducts.

PAH-DNA in Oral Cells and Oxidative DNA Damage in Oral and Mononuclear Cells by Immunoperoxidase Staining. PAH-DNA adducts in oral cells were assayed with an immunoperoxidase technique using polyclonal antiserum no. 1 (26) essentially as described previously (27). This antiserum has similar sensitivity and specificity to antiserum no. 29, which is in limited supply. 8OHdG was detected in oral and mononuclear cells using monoclonal antibody 1F7 (28) as described previously (29). Briefly, slides were treated with RNase A (100 μg/ml) at 37° for 1 h and with proteinase K (10 μg/ml) at room temperature for 7 min, and the DNA was denatured with 4N HCl for 7 min at room temperature. Bound primary antibodies (used at 1:200 dilution for antiserum no. 1 and 1:10 for 1F7) were detected with peroxidase-labeled ABC kits (Vector Laboratories, Burlingame, CA).

Quantitation of staining intensity was carried out on a Cell Analysis System (CAS 200) microscope (Becton Dickinson, San Jose, CA) with the Object Only program to determine

average absorbance in the nucleus for a minimum of 50 randomly selected cells. As a quality control, MCF7 cells were treated with or without 10 μg/ml benzo(*a*)pyrene diol epoxide (*NCI Chemical Carcinogen Repository, Midwest Research Institute, Kansas City, MO*) and stained with each batch of oral cells (CV, 24%; $n = 9$) for the PAH-DNA assay. MCF7 cells treated with aflatoxin B_1 were used as a positive control for the oxidative DNA assay (CV, 28%; $n = 14$).

Determination of Vitamins, Cotinine, and Cholesterol. α-Tocopherol and β-carotene were extracted into hexane after ethanol precipitation of the plasma proteins. Sample extracts were analyzed isocratically by reverse-phase HPLC as described (30). The laboratory accuracy of this analytical procedure, based on internally and externally prepared specimens, is $<\pm 4\%$ for α-tocopherol and $<\pm 8\%$ for β-carotene, whereas the within-day and day-to-day precision has a CV of <0.04. Vitamin C quantitation was carried out spectrophotometrically with 2,4-dinitrophenylhydrazine as a chromagen (31). The laboratory accuracy of this analytical procedure based on internally and externally prepared specimens is $<\pm 4\%$ whereas the day-to-day and within-day precision has a CV of <0.05.

Cholesterol and Cotinine Assays. Total cholesterol and urinary creatinine measurements were performed using commercially available diagnostic kits (Sigma Chemical Co.). Urinary cotinine levels were measured using an ELISA (STC Technologies, Bethlehem, PA). This assay can detect levels >50 ng/ml and was used to monitor for potential changes in smoking habits.

***GSTM1* Genotyping.** DNAs were analyzed for *GSTM1* genotype by PCR, essentially as described previously (32), using β-globin as an internal standard.

Statistical Analysis. The primary end points for this study were changes in the number of PAH-DNA adducts and in the level of 8OHdG in mononuclear and oral cells from the baseline to the three follow-up visits at 1 month, 3 months, and 6 months after commencement of treatment. For each subject, we computed a set of change scores defined as the change in response measurements from baseline to each follow-up time. The distribution of the original time-specific measurements and change scores were examined using graphic techniques (such as the histogram and box plots).

As in earlier studies, the distribution of the number of PAH-DNA adducts by ELISA in mononuclear cells was found to be highly skewed; therefore, we analyzed change scores for the log-transformed values as the end points for this study. Because a substantial number of subjects had nondetectable PAH-DNA adduct levels, we conducted confirmatory analyses treating the number of adducts as a binary variable (detectable *versus* nondetectable).

We began the comparative portion of the analysis by computing the mean and median change scores for each treatment group and comparing the groups at each time point via Mann-Whitney rank-sum test. Formal analyses compared change scores via linear regression, adjusting for two primary covariates: treatment group (coded as 1 for vitamin, 0 for placebo) and baseline adduct level (in the style of analysis of covariance). Initial regression models considered each follow-up time separately. More comprehensive final models included measurements from all follow-up times, relying on random effects modeling techniques to adjust for intrasubject correlation across visits (33). Additional regression models were fitted, incorporating predictor variables that may act as confounders, including age, gender, urinary cotinine, race/ethnic group, and *GSTM1* genotype. Treatment group by time interaction terms were also included in some regression models to assess whether the slope in change scores over time varied by treatment group.

Confirmatory analyses for the PAH-DNA adducts end point used the binary end point (detectable adducts *versus* nondetectable) as the outcome of interest. Initial logistic models regressed adduct detectability on treatment group and baseline detectability separately by time point. Final models considered measurements made at all time points simultaneously, accounting for intrasubject correlation by the use of generalized estimating equation methods (34). All generalized estimating equation models fitted used the logit link, binomial variance function, and exchangeable correlation structure.

Table 1 Baseline demographic characteristics of study participants who completed at least one follow-up visit

	Vitamin group		Placebo group	
	N	%	*N*	%
Sex				
Male	35	65	28	52
Female	19	35	26	48
Race/ethnicity				
African-American/Black	28	52	34	63
European-American/White	10	19	11	20
Hispanic/Latino	14	26	8	15
Other	2	4	1	2
Education				
0–12 yr	22	41	25	46
Post-high school technical	8	15	9	17
College	18	33	16	30
Graduate study	3	6	2	4
Marital status				
Never married	18	33	21	39
Married	16	30	13	24
Divorced/separated	16	30	14	26
Widowed	4	7	5	9
Employment status				
Employed	14	26	14	26
Housewife/child-care provider	6	11	4	7
Disability/retired	14	26	14	26
Unemployed	17	31	19	35
Student	3	6	3	6
Mean age (SD)	41.8 (8.5)		42.0 (9.0)	

Results

Preliminary Analyses. A total of 170 subjects were screened and 121 were randomized, 60 to treatment and 61 to placebo (Fig. 1). Of these, 112 completed at least one follow-up visit and therefore had measurable changes from baseline. Table 1 shows the demographic characteristics of the study participants who completed at least one follow-up visit. Although the vitamin and placebo groups differed slightly in demographic characteristics, they did not differ in smoking behavior. The mean age for smoking initiation and the mean number of cigarettes smoked per day were 15.6 ± 3.9 and 26.3 ± 6.0 in the vitamin group and 15.4 ± 4.0 and 26.1 ± 5.5 in the placebo group, respectively. Dropout rates were high in both groups but were higher in the placebo (53%) than treatment (35%) group (Fig. 1). A total of 13 study participants (6 vitamin group, 7 placebo group) were ≥1 month late for a study visit; of these, one was late for two visits.

Fig. 2 presents the median levels of vitamins and creatinine-adjusted urinary cotinine by treatment group at the four

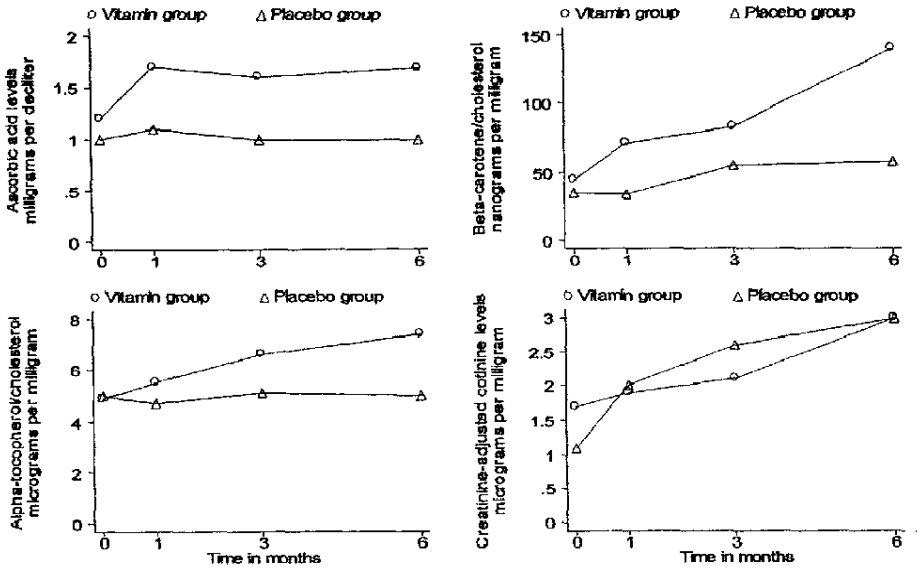

Fig. 2. Median plasma vitamin levels and creatinine-adjusted cotinine levels *versus* time (in months) from baseline visit.

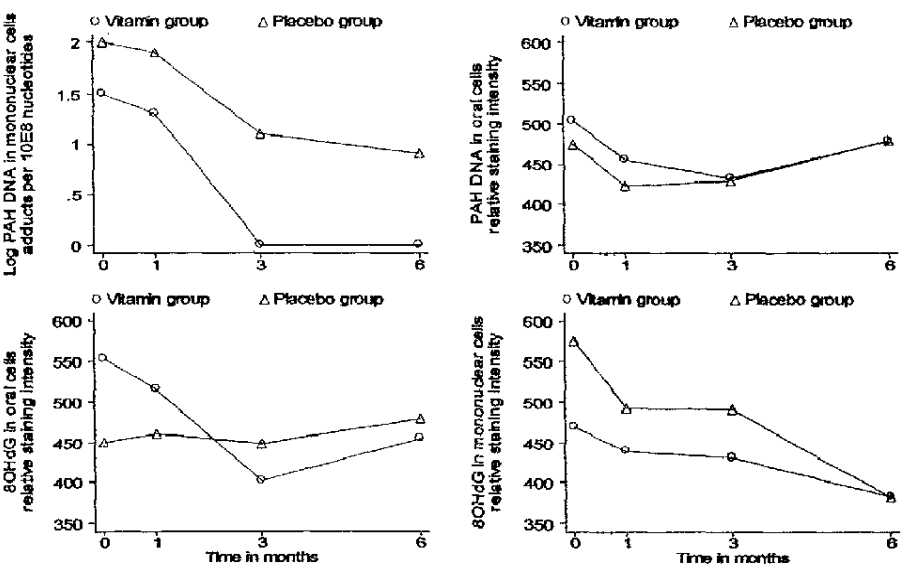

Fig. 3. Median response measurements *versus* time (in months) from baseline visit.

time points for subjects who provided at least one follow-up sample. Similarly, Fig. 3 presents the median levels of selected markers of DNA damage over time for the vitamin and placebo groups. Despite randomization, the vitamin and placebo groups differed significantly at baseline in median log-transformed levels of PAH-DNA adducts in mononuclear cells ($P = 0.02$) but not in the other end points or in plasma vitamin levels. Whereas vitamin levels increased only in the vitamin group, DNA damage levels decreased in both the vitamin group and the placebo group. Smoking habits, as measured by creatinine-adjusted urinary cotinine, increased over the time frame of the study.

Table 2 shows median changes from baseline to each of the three follow-up visits. Median changes in vitamin levels were consistently positive for all three vitamins in the vitamin group; between-group differences were significant at almost all time points. Median changes in all measures of DNA damage were negative in both the vitamin and placebo groups at almost all time points; none of the between-group differences was statistically significant.

Modeling Results. Linear regression modeling of the change in log-transformed PAH-DNA adducts in mononuclear cells revealed no statistically significant differences between the vitamin and placebo groups, whether looking at each visit separately or at all visits combined. After adjusting for the baseline PAH-DNA measurement, the mean difference in the log-transformed number of adducts was small and negative at

Table 2 Median changes from baseline in plasma vitamins, DNA damage, and urinary cotinine by treatment group

Variable	Vitamin group	Placebo group	Between-group P^a
Plasma vitamin C (mg/dl)			
Baseline to 1 mo	0.27[b]	−0.05	0.052
Baseline to 3 mo	0.42[b]	−0.08	0.011
Baseline to 6 mo	0.35[c]	−0.06	0.119
Plasma β-carotene/cholesterol (ng/mg)			
Baseline to 1 mo	13[b]	−4	0.017
Baseline to 3 mo	27[b]	−6	0.011
Baseline to 6 mo	107[b]	+4	0.001
Plasma α-tocopherol/cholesterol (μg/mg)			
Baseline to 1 mo	1.17	−0.35[c]	0.013
Baseline to 3 mo	1.51	−0.02	0.024
Baseline to 6 mo	2.41[b]	+0.33	0.001
Log PAH-DNA adducts in mononuclear cells (adducts/10^8 nucleotides)			
Baseline to 1 mo	0.00	−0.09	0.78
Baseline to 3 mo	−0.11[c]	−0.57[b]	0.57
Baseline to 6 mo	−0.03[c]	−0.34	0.94
PAH-DNA adducts in oral cells (relative staining intensity)			
Baseline to 1 mo	−31[b]	−16	0.068
Baseline to 3 mo	−45[b]	−28	0.290
Baseline to 6 mo	−45[b]	−39	0.463
8OHdG in oral cells (relative staining intensity)			
Baseline to 1 mo	−10	−30	0.28
Baseline to 3 mo	−55[b]	−35	0.19
Baseline to 6 mo	−27	−14	0.66
8OHdG in mononuclear cells (relative staining intensity)			
Baseline to 1 mo	−5	−74	0.17
Baseline to 3 mo	−48	−1	0.44
Baseline to 6 mo	−15	22	0.85
Urinary cotinine/creatinine (μg/mg)			
Baseline to 1 mo	−0.005	0.243[c]	0.029
Baseline to 3 mo	0.058	0.033	0.588
Baseline to 6 mo	−0.069	0.341[c]	0.143

[a] P from the Mann-Whitney two-sample test of the equality of median values.
[b] $P < 0.01$ for change from baseline by Wilcoxon's signed rank-sum test for paired samples.
[c] $P < 0.05$ for change from baseline by Wilcoxon's signed rank-sum test for paired samples.

each follow-up visit and overall (Table 3), indicating that the adduct levels decreased slightly more in the vitamin group compared with the placebo group. Because the analysis was conducted on the log-transformed values, these results can be back-transformed to show how the adduct levels changed on the original scale. Exponentiating the regression coefficient for treatment from the model gives an estimate of the ratio of the geometric means of the adduct levels in the vitamin group *versus* the placebo group. Combining all follow-up visits, the level of adducts in the vitamin group is 88% as large, on average, as the level in the placebo group. Subsequent models assessed whether the treatment effect varied over time; these results did not provide evidence of a time-by-treatment interaction. Additional models were fitted to see whether adjustment for age, gender, urinary cotinine levels, race/ethnic group, and *GSTM1* genotype would affect the estimated treatment effect. The parameter values reported in Table 4 clearly show the same patterns as those in Table 3. Thus, results were not modified by adjustment for potential confounders. Finally, we studied whether the effect of treatment varied by *GSTM1* status of the individual, as has been reported in other studies (12, 35). We found no evidence of this type of effect modification in the study sample ($P = 0.56$; data not shown).

Analysis of PAH-DNA in oral cells yielded similar results to those observed for mononuclear cells. At each follow-up visit, the mean change in adduct levels was not significantly different between the vitamin and placebo groups (Table 3). Combining information over all time points, the vitamin group displayed, on average, mean adduct levels about 35 units lower than those of the placebo group (not significant). We did not observe a significant time-by-treatment interaction. In addition, adjustment for additional confounders (age, gender, etc.) did not have a substantial impact on the estimated treatment effect for oral cell DNA damage (Table 4). However, a marginally significant interaction between *GSTM1* genotype and treatment ($P = 0.087$) was detected. Additional exploration of this result revealed that the vitamin treatment appeared to lower the number of adducts in oral cells by about 67 units ($P = 0.012$) in the *GSTM1*-positive subjects but actually appeared to increase adduct levels slightly (not significant) in the *GSTM1*-negative subjects.

Assessment of the 8OHdG measurements in oral and mononuclear cells revealed no significant effect of treatment in either the visit-specific or visits-combined analyses, whether adjusted for baseline measurement only or for the more extensive list of potential confounders (Tables 3 and 4). Furthermore, the treatment effect did not appear to vary over time (data not shown). As in the analysis of PAH-DNA in oral cells, however, we did observe a statistically significant difference in the treatment effect on 8OHdG in oral cells among subjects who are *GSTM1*-positive *versus* those who are *GSTM1*-negative ($P = 0.038$). Among the *GSTM1*-positive subjects, the mean change in 8OHdG for vitamin-treated subjects was estimated to be ~−41 units ($P = 0.18$). The corresponding figure among *GSTM1*-negative subjects was +51 units ($P = 0.12$), indicating a reversal of the treatment effect in this subgroup. Unlike the oral cell PAH-DNA analysis, the treatment effect was nonsignificant in both subgroups. For 8OHdG in mononuclear cells, we found no difference in treatment effects among *GSTM1*-positive and -negative subjects.

Confirmatory analyses were conducted, treating the PAH-DNA adduct levels in mononuclear cells as dichotomous (detectable *versus* nondetectable). These results failed to show a significant main effect of treatment on the presence of DNA adducts. Visit-specific analyses showed that the odds of having detectable adduct levels were about 1.3 to 2.0 times higher in the placebo group than in the vitamin group. The odds ratio over all time points was estimated to be ~1.7 ($P = 0.14$; 95% CI, 0.8–3.6), adjusting for visit number and baseline detectability. After adjusting for age, gender, race, cotinine level, and *GSTM1* status, the odds ratio increased slightly to 1.9 ($P = 0.15$; 95% CI, 0.8–4.6). Exploratory analysis revealed a statistically significant interaction between baseline detectability and treatment group ($P = 0.035$). Specifically, the vitamin treatment appeared to have little effect among those subjects with detectable PAH-DNA adduct levels at baseline. Among subjects with nondetectable adduct levels at baseline, the placebo group's adduct levels tended to become detectable over time, whereas the vitamin group's did not (data not shown). This finding may warrant additional inquiry in subsequent studies.

Table 5 compares the characteristics of subjects who remained on study for 6 months to those who left the study before the 6-month visit. In addition to being more likely to belong to the placebo group, subjects who dropped out were significantly younger and had higher levels of PAH-DNA at baseline than those who remained on study.

Table 3 Linear regression analysis of each response variable as a function of treatment group and baseline measurement

Results presented separately for each time point and for all time points combined.

	Mean difference (95% CI[a]) between vitamin group and placebo group in change from baseline measurement				
	1 mo	3 mo	6 mo	Over all time points combined[b]	*P* for combined analysis
PAH-DNA in mononuclear cells					
Log-transformed number	−0.15	−0.12	−0.003	−0.13	0.35
	(−0.50, 0.20)	(−0.54, 0.29)	(−0.46, 0.46)	(−0.41, 0.15)	
Ratio scale (vitamin to placebo)	86%	89%	100%	88%	
	(61%, 122%)	(58%, 134%)	(63%, 158%)	(66%, 116%)	
PAH-DNA in oral cells	−30	−44	−25	−35	0.09
	(−66, 5)	(−100, 12)	(−89, 40)	(−75, 5)	
8OHdG in oral cells	20	−32	−25	−7	0.73
	(−41, 81)	(−90, 27)	(−76, 26)	(−48, 34)	
8OHdG in mononuclear cells	−4	−81	−107	−56	0.19
	(−107, 98)	(−195, 33)	(−276, 62)	(−140, 28)	

[a] CI, confidence interval.
[b] Analysis over all time points combined is also adjusted for visit number.

Table 4 Linear regression analysis of each response variable as a function of treatment group, baseline measurement, age at baseline, gender, urinary creatinine-adjusted cotinine level, race/ethnicity, and *GSTM1* genotype

Results presented separately for each time point and for all time points combined.

	Mean difference (95% CI[a]) between vitamin group and placebo group in change from baseline measurement				
	1 mo	3 mo	6 mo	Over all time points combined[b]	*P* for combined analysis
PAH-DNA in mononuclear cells					
Log-transformed number	−0.20	−0.11	−0.003	−0.16	0.31
	(−0.57, 0.17)	(−0.58, 0.35)	(−0.56, 0.55)	(−0.46, 0.15)	
Ratio scale (vitamin to placebo)	82%	90%	100%	85%	
	(56%, 119%)	(56%, 142%)	(57%, 173%)	(63%, 116%)	
PAH-DNA in oral cells	−40	−46	−3	−34	0.11
	(−79, −1)	(−104, 11)	(−72, 66)	(−75, 8)	
8OHdG in oral cells	33	−18	−29	2	0.91
	(−33, 100)	(−80, 45)	(−86, 28)	(−40, 45)	
8OHdG in mononuclear cells	−12	−96	−80	−63	0.14
	(−130, 106)	(−200, 8)	(−261, 101)	(−147, 20)	

[a] CI, confidence interval.
[b] Analysis over all time points combined is also adjusted for visit number.

Discussion

In this chemoprevention trial, DNA damage was used as an intermediate or surrogate end point. The validity of surrogate end point markers depends on the extent to which the marker is a necessary event in the causal pathway to cancer (36). DNA damage is generally considered a necessary step in cancer initiation and is being used extensively in intervention studies (reviewed in Refs. 37 and 38). We used PAH-DNA adducts and oxidative DNA damage to determine the efficacy of a mixture of antioxidant vitamins in decreasing the effects of cigarette smoking. This study was initiated before the deleterious effects of high-dose β-carotene in smokers were reported. As these reports became available, we evaluated our protocol for potential changes in treatment. The decision was made to continue using the antioxidant mixture because it contained lower doses of β-carotene than had been shown to be deleterious and because study participants would receive it for only 6 months.

Vitamin levels increased significantly in subjects in the treatment group and not in the placebo group. All measures of DNA damage decreased in both groups, although median changes from baseline were more frequently significant in the vitamin group than in the placebo group (Table 2). We initially suspected that the large placebo effect was attributable to a reduction in cigarette smoking among subjects during their study participation. Before enrollment, our Institutional Review Board required us to counsel subjects on the hazards of smoking and to recommend that they quit. Only subjects who indicated they had no interest in quitting were recruited. However, after observing the changes in DNA damage in the placebo group, we analyzed urinary cotinine in all subjects. Creatinine-adjusted urinary cotinine levels did not decline in either group during study participation (Fig. 2 and Table 2) and were not correlated with adduct levels (data not shown). Although cotinine is only a short-term marker of smoking status, it indicates that changes in smoking behavior are not responsible for the observed decreases in DNA damage.

Placebo and Hawthorne effects are well known but not well understood (39). How subjects changed their life-style (diet, smoking habit, etc.) during the study, and which changes account for the decreased DNA damage, are not known. A previous study using a very similar mixture of antioxidants, also observed a placebo effect. Subjects on placebo had a 35% decrease in mononuclear cell DNA oxidized pyrimidines measured by the "comet" assay (40). However in that study, subjects

Table 5 Analysis of study dropouts comparing characteristics of those who remained on study for 6 months with those who left the study before the 6-month visit

	Subjects who dropped out before the 6-mo visit $N = 49$	Subjects who remained through the 6-mo visit $N = 63$	P^a
Age (mean)	39.7	43.9	0.008
% in vitamin group	41%	59%	0.060
% male	59%	56%	NS
Ascorbic acid at baseline (median)	1.17	1.09	NS
β-carotene at baseline (median)	0.067	0.037	NS
α-tocopherol at baseline (median)	5.00	4.65	NS
Log PAH-DNA in mononuclear cells at baseline (median)	2.13	1.34	0.007
PAH-DNA in oral cells at baseline (median)	361	502	0.071
8OHdG in oral cells at baseline (median)	481	513	NS
8OHdG in mononuclear cells at baseline (median)	487	474	NS

[a] From Student's t test for comparison of means, Pearson's χ^2 test for comparison of proportions, and the Mann-Whitney rank-sum test for comparison of medians.

on the combined antioxidants (100 mg vitamin C, 280 mg vitamin E and 25 mg β-carotene per day) had a 65% decrease in oxidized bases, which was significantly different from that in the placebo group. Other studies using the comet assay on mononuclear cells from subjects given vitamins C or E or β-carotene have found positive effects (41, 42); but other results were negative (43).

[^{32}P] Postlabeling has also been used to determine the effects of antioxidants. Gastric mucosa from subjects on a vitamin C trial (44) and oral cells of reverse smokers of chutta (rolled tobacco) on a 1-year intervention using vitamin A, riboflavin, zinc, and selenium (45) had lower levels of damage with treatment. Ascorbic acid was found to prevent endogenous oxidative DNA damage as assessed by HPLC measurement of 8OHdG in sperm (46). A pro-oxidant effect of vitamin C has also been suggested (47). Supplementation with 500 mg of vitamin C lead to decreased levels of 8OHdG in mononuclear cells but increased levels of oxidized adenine. But these results have been questioned in terms of potential assay artifacts (48, 49). A recent study recruited individuals occupationally exposed to environmental tobacco smoke and administered an over-the-counter antioxidant formulation containing β-carotene, vitamin C, α-tocopherol, zinc, selenium, and copper (50). After a 60-day supplementation there was a 62% decrease in 8OHdG. However, this study did not include a placebo control group. Other studies of urinary excretion of 8OHdG did not find an effect of treatment with vitamin C or E, or with coenzyme Q10 (51, 52) or β-carotene (53).

Although the vitamin group experienced a greater change from baseline than the placebo group for all but one response-variable measurement, none of the between-group differences was significant, with or without adjustment for possible confounders (Tables 3 and 4). We did not control for diet or for specific environmental exposures, such as fuels, in these analyses, and it is possible that study participation motivated shifts to a healthier diet during the period of observation. During the run-in period (on placebo), vitamin levels rose and mononuclear cell PAH-DNA adduct levels fell in both vitamin and placebo groups (Table 6). However, the relative stability of the plasma vitamin levels in the placebo group during the rest of its study participation (Fig. 2) suggests that changes in dietary intake of antioxidants (or unreported use of supplementation) in the placebo group during their study participation do not account for the group's declining adduct levels.

In our previous cross-sectional study, high serum antioxidant levels were associated with low DNA adduct levels in subjects who were *GSTM1*-null but not in those who had the gene (12). In a second study, we also found that the association between β-carotene and PAH-DNA was significant only in *GSTM1*-null subjects (35). However, a study by others found no relationship between DNA adducts by postlabeling and vitamins nor an effect of *CYP1A1* or *GSTM1* genotype (54). Although, in the present study, subjects with and without the gene did not differ in treatment effects, the numbers of subjects in the subgroups may have been too small for an interaction of genotype with treatment to have been detectable. We did find a greater effect of treatment on adduct levels in oral cells among *GSTM1*-positive than among *GSTM1*-negative subjects; that is, the change from baseline was significant in the GSTM1 positive vitamin group but not in the GSTM1 negative vitamin group. This finding suggests that vitamin intervention might be effective in the subgroup of patients who are *GSTM1*-positive. However, this subgroup analysis was exploratory in nature and made use of only about half the data, and the finding conflicts with some previous data.

Table 6 Changes in PAH-DNA adduct and vitamin levels from screening to baseline in the vitamin and placebo groups

	Total sample	Placebo group	Vitamin group
Log PAH-DNA	−0.41	−0.07	−0.90
Ascorbic acid mg/dl	0.17	−0.02	0.28
β-Carotene/cholesterol, μg/mg	1	1	1
α-Tocopherol/cholesterol, μg/mg	0.48	0.69	0.16

A major limitation of this study is the high and differential dropout rate. Dropouts were significantly younger than continuing participants in the study; they had higher mononuclear cell PAH-DNA adduct levels but lower oral cell adduct levels than continuing participants (Table 5). Moreover, the dropout rate was higher in the placebo group than in the treatment group, although the difference was not statistically significant (Table 5). Some placebo group members may have dropped out because they guessed their treatment assignment and were disappointed. Some participants had come into the study with the expressed hope that they would receive vitamins, and several commented that they knew their assignment because the vitamin pills smelled different from, and more like commercially available vitamins than, the placebo pills. These observations suggest a pitfall of blinded randomized trials, particularly where study participants make repeated visits and spend time in a common waiting area. Individual bubble packaging of the study agents might help to deter waiting area unblinding and would facilitate tablet counts.

A number of study participants were >1 week late in keeping their study visit appointments; 13 were ≥1 month late for an appointment. Delayed visits were not associated with treatment assignment and did not affect results (data not shown).

In recruiting participants, we found that many, perhaps most, heavy smokers who were interested in the study were already taking antioxidant supplements. Despite the findings of the β-Carotene and Retinol Efficacy trial and α-Tocopherol and β-Carotene Cancer Prevention studies, smokers in general appear to believe that vitamins can reduce the health risks associated with smoking.

The limitations of the study (high differential dropout, baseline difference in PAH-DNA adducts in mononuclear cells and a large placebo effect), may account for its failure to show that vitamin supplementation can influence DNA damage levels. During the 1-month run-in period between screening and collection of baseline specimens, adduct levels fell and vitamin levels rose among the study participants overall (Table 6). These changes were greater among individuals subsequently assigned to the vitamin group than among those subsequently assigned to the placebo group. All study participants were given placebo pills to take during the run-in; hence the changes cannot be attributed to treatment. However, the overall decline in adduct levels during study participation suggests that DNA damage is preventable and demonstrates the feasibility of using these biomarkers as intermediate end points in intervention studies.

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